

Induction of Cross-resistance to ABCB1 Substrates in Venetoclax-resistant Human Leukemia HL60 Cells

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Abstract. *Background/Aim:* Resistance to venetoclax, a selective inhibitor of BCL2 apoptosis regulator (BCL2), is regarded as a clinical problem. However, it is unclear whether resistance to venetoclax induces cross-resistance to other drugs. *Materials and Methods:* Venetoclax-resistant HL60/VEN cells were newly established through continuous exposure of human acute promyelocytic leukemia HL60 cells to venetoclax, and drug sensitivity, apoptotic activity, and mRNA expression were compared between HL60 and HL60/VEN cells. *Results:* HL60/VEN cells displayed approximately 3-fold resistance to venetoclax, maintained their ability to synthesize DNA and had low apoptotic activity. HL60/VEN cells also exhibited diverse sensitivity to cytotoxic drugs, especially resistance to ATP binding cassette subfamily B member 1 (ABCB1) substrates, and up-regulation of ABCB1 mRNA. However, the sensitivity of HL60/VEN cells to venetoclax was not restored by ABCB1 inhibitor. ABCB1-overexpressing cells did not show resistance to venetoclax. *Conclusion:* HL60/VEN cells exhibited up-regulation of ABCB1 in addition to an alteration in apoptotic activity, and cross-resistance to ABCB1 substrates was clarified. However, sensitivity to venetoclax was hardly affected by ABCB1.

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Venetoclax is a first-in-class, oral, selective inhibitor of BCL2 apoptosis regulator (BCL2) that is approved worldwide (1, 2). Venetoclax as monotherapy or in combination with rituximab is an effective treatment, provides durable responses in adults with recurrent/refractory chronic lymphocytic leukemia, and has a manageable safety profile in pivotal clinical trials (3-5). Trials are currently underway to expand its use for various other types of cancer, including multiple myeloma and chronic myeloid leukemia (6, 7).

BCL2 an anti-apoptotic protein is a regulator of cell death (*i.e.*, apoptosis) (8). Evasion of apoptosis is one of the typical characteristics of cancer cells, and a change in expression of BCL2 family proteins has been reported in many cancer types (9-11). Therefore, current interest is focused on targeting BCL2 to treat various hematological malignancies, which are typically mediated by the intrinsic regulation of apoptosis or dysregulation of the mitochondrial pathway (12-14). Venetoclax binds and neutralizes BCL2 (BH3 domain), resulting in the release of cytochrome *c* from the mitochondria and subsequent activation of the intrinsic apoptotic pathway through a caspase cleavage cascade (8, 15, 16).

Multidrug resistance remains a significant problem in cancer chemotherapy (17, 18). Tumor cells have been found to acquire resistance not only to cytotoxic anticancer drugs, but also to molecular targeting agents. To date, the mechanisms of resistance to venetoclax have been investigated, and changes in molecular profiles have been found to occur in venetoclax-resistant cells (19). Although resistance to venetoclax is regarded as a clinical problem (20, 21), it is unclear whether resistance to venetoclax induces resistance to drugs besides venetoclax *i.e.*, leads to cross-resistance.

Here, a human acute promyelocytic leukemia cell line, HL60, as a leukemia cell model, was continuously exposed to venetoclax at a clinically achievable concentration, and a novel venetoclax-resistant cell (named as HL60/VEN) was established. The sensitivity to venetoclax and other anticancer drugs and the molecular changes in HL60/VEN cells were examined and compared with those in HL60 cells.

Materials and Methods

Chemicals. Venetoclax was purchased from Selleck Chemicals (Houston, TX, USA). Carboplatin, cisplatin, doxorubicin hydrochloride, etoposide, 5-fluorouracil, paclitaxel, vinblastine sulfate and vincristine sulfate were purchased from FUJIFILM Wako Pure Chemical Corp. (Osaka, Japan). 7-Ethyl-10-hydroxycamptothecin (SN-38, an active metabolite of irinotecan hydrochloride) was purchased from LKT Laboratories, Inc. (St. Paul, MN, USA). Mitoxantrone dihydrochloride was obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) and 1-methoxy-5-methylphenazinium methylsulfate were acquired from Dojindo Laboratories (Kumamoto, Japan).

Cells and cell culture. The human acute promyelocytic leukemia cell line HL60 (JCRB0085; Japanese Collection of Research Bioresources Cell Bank, National Institutes of Biomedical Innovation, Health and Nutrition, Osaka, Japan), which is used as a model of leukemia cells, was routinely cultured in medium consisting of RPMI1640 (Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Gibco™, Thermo Fisher Scientific K.K., Tokyo, Japan) and 50 U/ml penicillin-50 µg/ml streptomycin (Invitrogen™, Thermo Fisher Scientific). HL60 cells were grown in culture flasks in humidified air with 5% CO₂ at 37°C, and subcultured every 3 or 4 days.

The human cervical carcinoma cell line HeLa, selected as a model of cancer cells, was maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (Gibco™) and 100 mg/l of kanamycin sulfate (Invitrogen™) (22, 23). Cells were seeded into culture flasks, grown in a humidified atmosphere of 5% CO₂-95% air at 37°C, and subcultured with 0.05% trypsin-0.02% ethylenediaminetetra-acetic acid (Invitrogen™). HeLa/TXL and HeLa/SN100 cells were previously established by continuous exposure to paclitaxel or SN-38, respectively (22, 23). HeLa/TXL and HeLa/SN100 cells were maintained in a manner similar to HeLa cells, except that DMEM contained 20 nM of paclitaxel and 100 nM of SN-38, respectively.

Establishment of venetoclax-resistant HL60 sublines. Briefly, HL60 cells were cultured in RPMI1640 medium containing 1 µM venetoclax, as a sub-growth-inhibition concentration, for approximately 3 months. The cells stably growing in the presence of 1 µM venetoclax were isolated and then cloned by a colony-formation method using a RPMI1640 medium containing 0.75% methylcellulose. In the preliminary experiments, the characteristics of several clones were examined, and the selected clone was designated as HL60/VEN. HL60/VEN cells were maintained in a manner similar to HL60 cells, except that the RPMI1640 medium contained 1 µM venetoclax.

Cell proliferation assay. The effects of the test drugs on the cell proliferation of HL60 and HL60/VEN cells were evaluated by a WST-1 assay (22, 23). The cells (2.5×10³/well) were seeded into a 96-well plate in 50 µl of RPMI1640 medium without any drugs. After 24 h of pre-culture, each well was supplemented with 50 µl medium containing different concentrations of the test drugs. After incubation for 72 h at 37°C, 10 µl of WST-1 solution was added to the wells of the 96-well plates; 5 h later, the absorbance was measured at 450 nm with a reference wavelength of 620 nm using a Spectra Fluor microplate reader (Tecan Switzerland, Switzerland).

HeLa and its resistant cell line were seeded (1×10³/well) into 96-well plates in 100 µl of DMEM without any drugs. After a 24 h pre-culture, the medium was replaced with medium containing a test drug at different concentrations. After incubation for 72 h at 37°C, DMEM was replaced with 110 µl of medium containing WST-1 reagent (10 µl of WST-1 + 100 µl of DMEM); 3 h later, the absorbance was determined as mentioned above. The 50% growth-inhibitory concentration (IC₅₀) of the test drugs was estimated according to the sigmoid inhibitory effect model, $E = E_{\max} \times [1 - C^\gamma / (C^\gamma + IC_{50}^\gamma)]$, using the nonlinear least-squares fitting method (Solver, Microsoft® Excel), in which E and E_{max} represent the surviving fraction (% of control) and its maximum, respectively; C and γ represent the concentration in the medium and the sigmoidicity factor, respectively (22, 23).

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay. DNA synthesis as an index for growth activity was assessed using the BrdU incorporation assay (Cell Proliferation ELISA, BrdU (colorimetric); Roche Diagnostics K.K., Tokyo, Japan). Cells (5×10³/well) were seeded into 96-well plates in 50 µl of RPMI1640 medium without any drugs. After a 24 h pre-culture, 50 µl of medium containing 0.08 to 20 µM of venetoclax was added to each well, resulting in a final concentration of 0.04 to 10 µM of venetoclax; the cells were further incubated for 72 h. According to the manufacturer's instructions, the incorporation of BrdU was conducted 24 h after treatment with venetoclax. After removing the BrdU labeling reagents, cells were fixed, and the BrdU-labeled DNA was denatured. Samples were incubated for 90 min with an anti-BrdU monoclonal antibody conjugated with peroxidase, and the substrates for peroxidase were added to the samples. The absorbance of the samples was measured at 360 nm with a reference wavelength of 450 nm using a Spectra Fluor microplate reader (Tecan).

Flow cytometry. HL60 and HL60/VEN cells were treated with 5 or 10 µM venetoclax for 48 h. Thereafter, cells were harvested and labeled with fluorescein isothiocyanate (FITC)-conjugated annexin V (BioLegend, San Diego, CA, USA) and propidium iodide (PI, 50 µg/ml; FUJIFILM Wako) for detection of early and late apoptosis. FITC and PI fluorescence signals were detected using a FACSCalibur™ instrument (Becton Dickinson, Franklin Lakes, NJ, USA) (24, 25). Data were analyzed using CELLQuest Ver. 3.3 (Becton Dickinson) software. At least 10,000 cells were used in the analysis.

DNA fragmentation assay. As a common feature of apoptosis, DNA fragmentation after exposure to venetoclax was examined (26). HL60 and HL60/VEN cells were seeded onto plastic culture dishes (60 mm in diameter) in 5 ml of RPMI1640 medium. After cells were precultured for 24 h, 1, 5, 10, and 20 µM venetoclax was added to the cells which were incubated for a further 24 h in a humidified atmosphere containing 5% CO₂ at 37°C. Cells were then collected,

Table I. Sequences of oligonucleotide primers used in quantitative reverse transcription-polymerase chain reaction in this study.

Gene symbol	Encoded protein	Forward, 5'-3' orientation	Reverse, 5'-3' orientation
<i>ACTB</i>	β -Actin	TCATGAAGTGTGACGTGGACATC	TGCATCCTGTGCGCAATG
<i>BAK1</i>	BCL2 antagonist/killer 1	ATGGTCACCTTACCTCTGCAA	TCATAGCGTCGGTTGATGTCG
<i>BAX</i>	BCL2-associated X, apoptosis regulator	TGGCAGCTGACATGTTTTCTGAC	TCACCCAACCACCCTGGTCTT
<i>BCL2</i>	BCL2 apoptosis regulator	TCGCCCTGTGGATGACTGA	CAGAGACAGCCAGGAGAAATCA
<i>BCL2L12</i>	BCL2-like 12	CCCTCGGCCTTGCTCTCT	TCCGCAGTATGGCTTCCTTC
<i>BCL2L1</i>	BCL2-like 1	GCTTGGATGGCCACTTACCT	TCAGGAACCAGCGTTGAAG
<i>BCL2A1</i>	BCL2 related protein A1	ACACAGGAGAATGGATAAGGCA	TGGTCAACAGTATTGCTTCAGGA
<i>MCL1</i>	MCL1 apoptosis regulator, BCL2 family member	GGACAAAACGGGACTGGCTA	CAGCAGCACATTCCTGATGC
<i>ABCB1</i>	ATP-binding cassette subfamily B member 1	TTCCCTCACCCAGGCAATG	ATGAGTTTATGTGCCACCAAGTAG
<i>ABCC1</i>	ATP-binding cassette subfamily C member 1	CAGTGACCTCTGGTCCTTAAACAA	TTGGCGCATTCTTCTTCC
<i>ABCC2</i>	ATP-binding cassette subfamily C member 2	ACTGTGTGACATCGGTAGCATGGA	AAGAGGCAGTTTGTGAGGGATGA
<i>ABCG2</i>	ATP-binding cassette subfamily G member 2	TGACGGTGAGAGAAAACCTTAC	TGCCACTTTATCCAGACCT

and RNase and protein digestion enzyme solution were added using an Apoptosis Ladder Detection kit (FUJIFILM Wako). The DNA extracted from cells was electrophoresed on a 2% agarose gel. After electrophoresis, the agarose gels were stained with SYBR[®] green, and DNA fragmentation was detected by UV transillumination.

Quantitative reverse transcription-polymerase chain reaction (RT-PCR). After cells were precultured for 48 h, total RNA was extracted from the cells with a GenElute[™] Mammalian Total RNA Miniprep kit (Sigma-Aldrich). The mRNA expression levels of BCL2 antagonist/killer 1 (*BAK1*), BCL2-associated X apoptosis regulator (*BAX*), *BCL2*, BCL2-like 1 (*BCL2L1*), BCL2-like 12 (*BCL2L12*), BCL2-related protein A1 (*BCL2A1*), MCL1 apoptosis regulator, BCL2 family member (*MCL1*), ATP binding cassette subfamily B member 1 (*ABCB1*), *ABCC1*, *ABCC2* and *ABCG2* were measured by RT-PCR (27). Total RNA (500 ng) was used for RT reaction with a PrimeScript[™] RT reagent kit (Takara Bio Inc., Shiga, Japan) and a thermal cycler (i-Cycler; Bio-Rad Laboratories, Hercules, CA, USA). The RT reaction was conducted in 20 μ l of reaction buffer at 37°C for 15 min, and terminated by heating at 85°C for 5 s followed by cooling at 4°C. Real-time PCR was performed with a 7500 Fast Real-Time PCR system (Applied Biosystems, Waltham, MA, USA) and SYBR Premix Ex Taq[™] (Takara Bio). β -Actin (*ACTB*) was used as an internal standard. The primers were synthesized by GeneDesign, Inc. (Ibaraki, Japan) and their sequences are presented in Table I. To compare the relative target mRNA levels, the comparative threshold cycle method was used, as previously described (27).

Statistical analysis. Comparisons between two groups or among three groups were performed with Student's unpaired *t*-test or a non-repeated one-way analysis of variance followed by the Dunnett test, respectively, using JMP[®] Pro 15.2.0. (SAS Institute Japan Ltd., Tokyo, Japan). A *p*-value of less than 0.05 (two-tailed) was considered significant.

Results

Sensitivity to venetoclax. The IC₅₀ values for venetoclax in HL60 and HL60/VEN cells are indicated in Table II. Treatment with venetoclax for 72 h caused growth inhibition

of HL60 and HL60/VEN cells, with IC₅₀ values of 2.31 μ M and 6.26 μ M, respectively. Such findings indicate a 2.71-fold greater resistance of HL60/VEN cells relative to HL60 cells. The effects of venetoclax on the DNA incorporation of BrdU into HL60 and HL60/VEN cells were examined (Figure 1A). The incorporation of BrdU into DNA in HL60 cells was unchanged up to 0.625 μ M venetoclax, whilst significant suppression was observed at 2.5 and 10 μ M of venetoclax. However, BrdU incorporation in HL60/VEN cells was maintained up to 2.5 μ M venetoclax, although a significant increase was noted at 0.625 μ M venetoclax, and significant inhibition was observed at 10 μ M venetoclax.

DNA fragmentation and apoptosis induction by venetoclax.

Fragmentation of DNA by exposure to venetoclax was examined in HL60 and HL60/VEN cells (Figure 1B). DNA fragmentation was observed in both cell lines treated with venetoclax, but the onset of DNA fragmentation in HL60/VEN cells occurred at a higher concentration compared with that in HL60 cells.

The induction of apoptosis in HL60 and HL60/VEN cells was measured by FITC-annexin V/PI staining after treatment with venetoclax (Figure 1C and D). The percentage of early and late apoptosis after 24 h of venetoclax treatment concentration-dependently increased in HL60 cells. Although the numbers of early and late apoptotic cells in HL60/VEN cells also increased in a venetoclax concentration-dependent manner, their fraction was significantly lower in HL60/VEN cells than in HL60 cells.

Expression profiles of apoptotic regulating factors. The mRNA expression levels of apoptosis-related factors were examined by real-time quantitative RT-PCR. The mRNA level of *BCL2*, an apoptosis-inhibitory factor, was significantly lower in HL60/VEN cells than HL60 cells, resulting in an expression

Table II. Sensitivity of HL60 and HL60/VEN cells to different types of anticancer drugs. Each 50% growth-inhibitory concentration (IC₅₀) value represents the mean±standard deviation (n=8).

Drug	Unit	IC ₅₀ value		RR
		HL60	HL60/VEN	
Venetoclax	μM	2.31±0.12	6.26±0.81**	2.71
Vincristine	nM	11.2±2.65	23.0±6.02**	2.05
Vinblastine	nM	45.1±4.71	100±9.13**	2.10
Paclitaxel	nM	0.095±0.019	0.272±0.090**	2.70
Etoposide	μM	0.293±0.015	1.53±0.32**	5.28
Doxorubicin	nM	20.3±1.21	122±18.2**	5.99
SN-38	nM	4.21±0.32	4.93±0.73	1.17
Mitoxantrone	nM	15.6±0.69	23.8±1.20**	1.52
5-Fluorouracil	μM	37.1±6.58	46.6±9.10	1.25
Cisplatin	μM	3.04±0.27	3.06±0.32	1.00
Carboplatin	μM	17.1±1.39	27.3±2.22**	1.60

RR: Relative resistance, calculated as the IC₅₀ value for HL60/VEN cells divided by the value for HL60 cells. **Significantly different at p<0.01 from HL60 cells (unpaired Student's *t*-test).

ratio of 0.21 relative to that of HL60 cells (Figure 2A and C). However, the mRNA levels of *BCL2A1* and *MCL1*, which are also apoptosis-inhibitory factors, were significantly higher in HL60/VEN cells than HL60 cells, resulting in expression ratios of 1.79 and 1.33, respectively. There was no difference in the expression of *BCL2L1* and *BCL2L2* mRNAs between cell lines. In addition, there was no significant difference in the mRNA expression of the apoptosis-promoting factors *BAK1* and *BAX* (Figure 2B and C).

Sensitivity to different types of anticancer drugs. Table II summarizes the IC₅₀ values for different types of anticancer drugs against HL60 and HL60/VEN cells. The IC₅₀ values for vincristine, vinblastine, paclitaxel, etoposide, and doxorubicin, which are substrates for ABCB1, were significantly higher for HL60/VEN cells than HL60 cells. No significant difference in the sensitivity to SN-38, an active metabolite of irinotecan hydrochloride and substrate of ABCG2, was found between HL60 and HL60/VEN cells. However, the sensitivity to mitoxantrone, a substrate for ABCG2, was significantly reduced in HL60/VEN cells. There were no differences in sensitivity to 5-fluorouracil and cisplatin in either cell line; however, sensitivity to carboplatin was significantly lower in HL60/VEN cells.

Expression profiles of selected ABC transporters. Figure 3 shows the expression level of the representative ABC transporters, which are involved in resistance to various types of anticancer drugs. The expression level of *ABCB1* mRNA in HL60/VEN cells was significantly higher than that in HL60 cells, and that of *ABCG2* mRNA tended to be increased in

HL60/VEN cells. The levels of *ABCC1* and *ABCC2* mRNAs were significantly reduced in HL60/VEN cells.

Participation of ABCB1 in the resistance to venetoclax. To clarify the participation of ABCB1 in the resistance of HL60/VEN cells to venetoclax, the effect of cyclosporine A, an inhibitor of ABCB1, on sensitivity to venetoclax was determined (Table IV). The IC₅₀ values for venetoclax against HL60 and HL60/VEN cells in the presence of 1 μM cyclosporine A were comparable to the respective IC₅₀ values in the absence of cyclosporine A.

Sensitivity to venetoclax in ABCB1- or ABCG2-overexpressing cells. The sensitivity to venetoclax was examined in the ABCB1-overexpressing HeLa/TXL and ABCG2-overexpressing HeLa/SN100 cells, which were previously established via continuous exposure to paclitaxel or SN-38 (22, 23). The IC₅₀ values for venetoclax were comparable between HeLa and the ABCB1-overexpressing HeLa/TXL cells (Table III). The IC₅₀ value for venetoclax against ABCG2-overexpressing HeLa/SN100 cells was significantly lower than those in HeLa cells.

Discussion

Herein, venetoclax-resistant cells were newly established through the continuous exposure of HL60 cells, a leukemia cell model, to 1 μM venetoclax (clinically achievable plasma concentration); these cells were designated as HL60/VEN cells. Compared with HL60 cells, HL60/VEN cells showed approximately 3-fold resistance to venetoclax in the cell growth-inhibition assay (Table II). BrdU incorporation was maintained at a higher concentration of venetoclax in HL60/VEN cells than in HL60 cells (Figure 1A), implying that HL60/VEN cells exhibited resistance to venetoclax from the viewpoint of their ability to synthesize DNA. Based on these findings, the established HL60/VEN cells were characterized as venetoclax-resistant, and were demonstrated to be a useful tool for identifying the potential mechanisms of resistance.

The induction of apoptosis by venetoclax was lower in HL60/VEN cells than HL60 cells based on annexin V staining and DNA fragmentation assay (Figure 1B-D). These findings indicate a change in the apoptotic pathway of HL60/VEN cells, suggesting that this alteration was at least involved in the mechanism of resistance to venetoclax in HL60/VEN cells. The mRNA expression levels of the BCL2 family proteins were examined by real-time RT-PCR. The mRNA expression of *BCL2* was significantly lower in HL60/VEN cells, whereas that of *BCL2A1* and *MCL1* increased significantly (Figure 2A). Some studies reported that a low or lack of expression of *BCL2* induced high resistance to venetoclax, consistent with the characteristics of HL60/VEN cells, although the examined cell lines differed from those used in this study (28-30). Previous

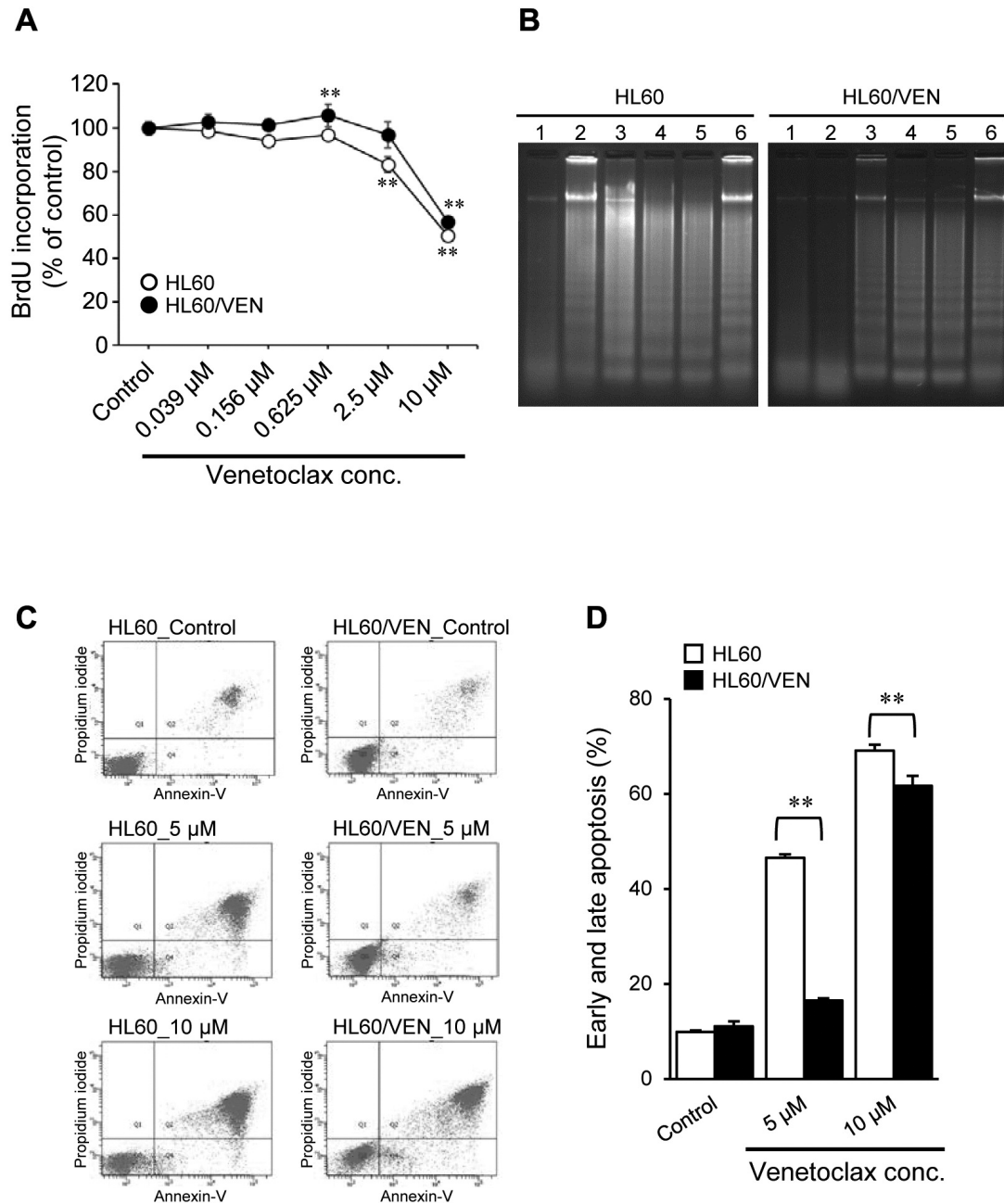
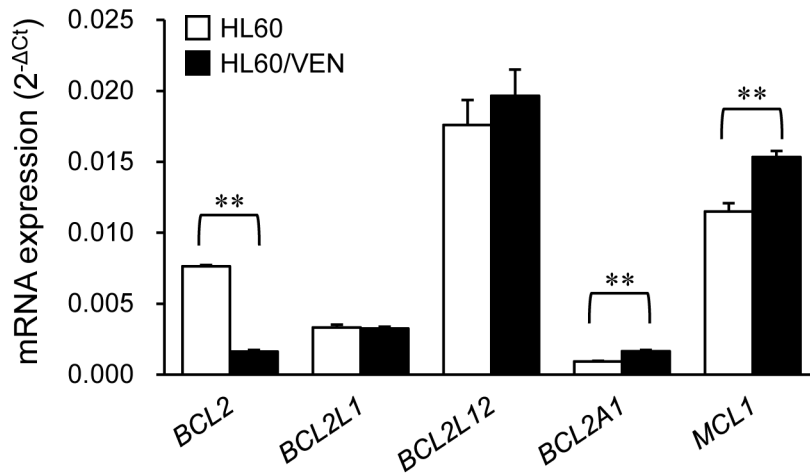
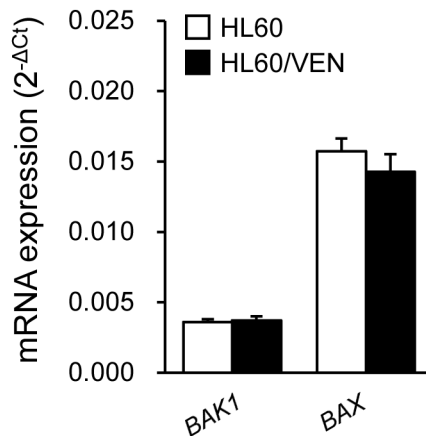


Figure 1. Molecular characteristics of HL60 and HL/VEN cells. **A**: Incorporation of 5-bromo-2'-deoxyuridine (BrdU) into DNA by HL60 and HL60/VEN cells. Cells were treated with venetoclax at 0.04 to 10 μM for 72 h, and the incorporation of BrdU was conducted for 24 h. BrdU-labeled DNA were determined colorimetrically using a cell proliferation enzyme-linked immunosorbent assay. Each bar shows the mean \pm standard deviation ($n=4$). **Significantly different at $p<0.01$ from the respective control (non-repeated analysis of variance followed by Dunnett test). **B**: Electropherograms of DNA fragmentation after exposure to venetoclax in HL60 and HL60/VEN cells. HL60 and HL60/VEN cells were treated with 1, 5, 10, and 20 μM venetoclax for 24 h. The extracted DNA from cells was electrophoresed by 2% agarose gel, and were stained by SYBR[®] green. The lane identifications were as follows. Lane 1: Control (no treatment with drug); lane 2: 1 μM venetoclax; lane 3: 5 μM venetoclax; lane 4: 10 μM venetoclax treatment; Lane 5: 20 μM venetoclax; Lane 6: Positive control (treatment with 1 μM cisplatin). **C**: Flow cytometry-based detection of apoptosis by concurrent staining with annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI). HL60 and HL60/VEN cells were treated with 5 or 10 μM venetoclax for 48 h. Cells were labeled with FITC-conjugated annexin V and PI for early and late apoptosis detection. Cells were regarded as follows: Stained with neither annexin V nor PI: live cells (Q3); stained only with annexin V: early apoptotic cells (Q4); and stained with both annexin V and PI: late apoptotic cells (Q2). **D**: Quantification of flow cytometric data showing the proportion of apoptotic cells (early plus late apoptosis). **Significantly different at $p<0.01$ from the respective HL60 cells (unpaired Student's *t*-test).

A Apoptosis-inhibitory factors



B Apoptosis-promoting factors



C Expression ratio

Gene	Ratio
BCL2	0.21
BCL2L1	0.98
BCL2L12	1.12
BCL2A1	1.79
MCL1	1.33
BAK1	1.03
BAX	0.91

Figure 2. mRNA expression profile of apoptosis-regulating factors in HL60 and HL60/VEN cells. Total RNA was extracted from HL60 and HL60/VEN cells using a spin column method. The mRNA expressions of target gene and β -actin, as an internal standard, were evaluated by real-time reverse transcription-polymerase chain reaction using a SYBR[®] green. To compare the relative expression of target mRNA levels, the comparative threshold cycle method was used. A: Expression of apoptosis-inhibitory factors BCL2 apoptosis regulator (BCL2), BCL2-like 1 (BCL2L1), BCL2-like 12 (BCL2L12), BCL2-related protein A1 (BCL2A1) and MCL1 apoptosis regulator, BCL2 family member (MCL1). B: Expression of apoptosis-promoting factors BCL2 antagonist/killer 1 (BAK1) and BCL2-associated X apoptosis regulator (BAX). C: The relative expression ratio was calculated as the expression level ($2^{-\Delta C_t}$) in HL60/VEN cells divided by that in the respective HL60 cells. Each bar represents the mean \pm standard deviation ($n=4$). **Significantly different at $p<0.01$ from the respective HL60 cells (unpaired Student's t -test).

reports also revealed a high expression of *MCL1* in venetoclax-resistant cells, which is similar to that found in HL60/VEN cells. Esteve-Arenys *et al.* (31, 32) reported that the accumulation of BCL2-related protein A1 is a major mechanism involved in acquired resistance to venetoclax; findings of the present study were comparable with this. Recently, Haselager *et al.* (33) demonstrated that BCL2-like 1 (*BCL2L1*) was also important in venetoclax resistance in chronic lymphocytic leukemia cells;

however, the mRNA expression of *BCL2L1* was found to be comparable between HL60 and HL60/VEN cells in this study (Figure 2A). Such findings suggest complicated changes in expression of BCL2 family members owing to venetoclax resistance. Collectively, our findings suggest that HL60/VEN cells had the typical characteristics of venetoclax resistance. Investigating other potent mechanisms would thus be meaningful for reversing this resistance.

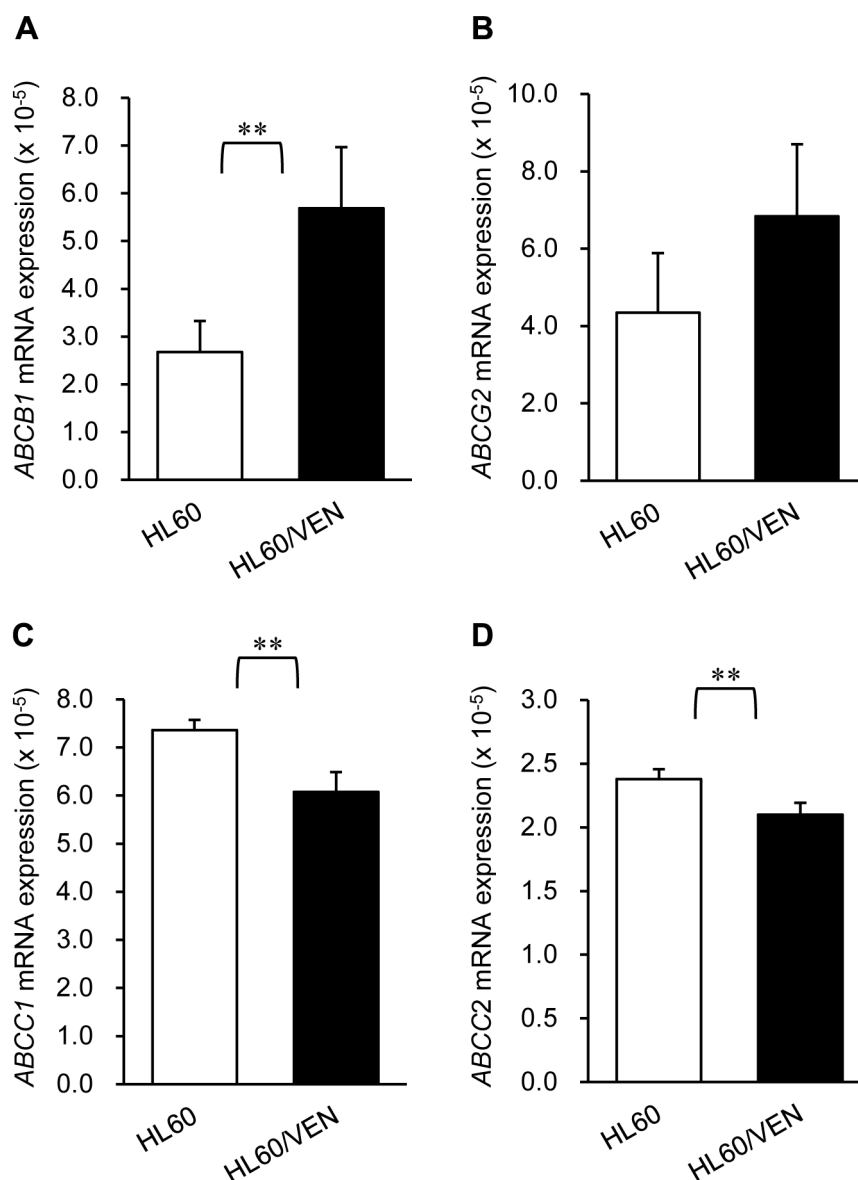


Figure 3. mRNA expression profiles of ATP-binding cassette subfamily B member 1 (*ABCB1*) (A), *ABCG2* (B), *ABCC1* (C), and *ABCC2* (D) in HL60 and HL60/VEN cells. Total RNA was extracted from HL60 and HL60/VEN cells using a spin column method. The mRNA expressions of target gene and β -actin, as an internal standard, were evaluated by real-time reverse transcription-polymerase chain reaction using a SYBR[®] green. To compare the relative expression of target mRNA, the comparative threshold cycle method was used. Each bar represents the mean \pm standard deviation (n=4). **Significantly different at $p < 0.01$ from the respective HL60 cells (unpaired Student's *t*-test).

Interestingly, HL60/VEN cells showed diverse sensitivity to cytotoxic anticancer drugs, in particular, resistance to *ABCB1* substrates (Table II). This resistance is supported by the up-regulation of *ABCB1* mRNA in HL60/VEN cells compared with HL60 cells (Figure 3). However, sensitivity to venetoclax in HL60/VEN cells was not restored by cyclosporine A, a representative *ABCB1* inhibitor, nor did it change the IC_{50} against HL60 cells (Table IV). Moreover,

ABCB1-overexpressing HeLa cells, established by continuous exposure to paclitaxel (22), showed comparable sensitivity to venetoclax relative to HeLa cells (Table III). These findings suggest that up-regulation of *ABCB1* does not contribute to venetoclax resistance in HL60/VEN cells but elicits a cross-resistance to *ABCB1* substrates. The lack of contribution of *ABCB1* to venetoclax resistance may be explained by the lower binding affinity of venetoclax for

Table III. Sensitivity to venetoclax in ATP-binding cassette subfamily B member 1 (ABCB1)-overexpressing (HeLa/TXL) and ABCG2-overexpressing (HeLa/SN100) cells. Each value for the 50% growth-inhibitory concentration (IC₅₀) represents the mean±standard deviation (n=8 or 16).

Cell line	IC ₅₀ value (µM)	RR
HeLa	19.7±3.82	-
HeLa/TXL	19.4±3.26	0.98
HeLa/SN100	16.1±1.49*	0.82

RR: Relative resistance, calculated as the IC₅₀ value for HeLa/TXL or HeLa/SN100 cells divided by that for HeLa cells. *Significantly different at p<0.05 from HeLa cells (non-repeated analysis of variance followed by Dunnett test).

Table IV. Effects of cyclosporine A (CyA) on the sensitivity to venetoclax of HL60 and HL60/VEN cells. Each value for the 50% growth-inhibitory concentration (IC₅₀) represents the mean±standard deviation (n=8).

Cells	IC ₅₀ value (µM)		RS
	Control	+ 1 µM CyA	
HL60	2.86±0.19	3.04±0.39	0.94
HL60/VEN	7.33±1.16	7.36±2.02	1.00

RS: Relative sensitivity, calculated as the IC₅₀ value for the respective control divided by that for cells treated with 1 µM CyA.

ABCB1 than other cytotoxic anticancer drugs, e.g., vincristine and doxorubicin. In fact, the IC₅₀ values of ABCB1-overexpressing murine monocytic leukemia P388/dx cells for in the transport assays using calcein, which is a substrate for ABCB1, were approximately 30 µM for venetoclax and approximately 2.9 µM for verapamil, a representative ABCB1 inhibitor (34).

Of note, the induction of ABCB1 mRNA by continuous exposure to venetoclax was observed in this study (Figure 3). However, Weiss *et al.* (34) reported the suppression of ABCB1 mRNA expression by venetoclax in LS180 human colon carcinoma cells, which is the standard model for investigating pregnane X receptor-mediated induction. The reason for this difference remains unclear; however, other mechanisms, other than those mediated by pregnane X receptor, may contribute to the induction of ABCB1 in HL60 cells. In any case, the variation in expression of ABCB1 induced by venetoclax was suggested to be clinically meaningful and a problem, leading to ABCB1-mediated multidrug resistance or drug–drug interaction.

By employing ABCG2-overexpressing cancer cells, Wang *et al.* (35) found that venetoclax potentiated the

efficacy of ABCG2 substrate drugs by blocking ABCG2 efflux function and inhibiting the ATPase activity of ABCG2. The researchers also demonstrated that the cytotoxicity of venetoclax was similar between ABCG2-mediated resistant cells and their parental cells, and that venetoclax did not affect the protein expression of ABCG2. The mRNA expression of ABCG2 tended to increase in HL60/VEN cells (Figure 3) and the IC₅₀ value for mitoxantrone but not SN-38, both of which are ABCG2 substrates, was significantly high in HL60/VEN cells (Table II). However, the ABCG2-overexpressing HeLa/SN100 cells, established by continuous exposure to SN-38 (23), displayed higher sensitivity to venetoclax than its parental cell line (Table III), although the relative resistance was not large (0.82). The reasons for these complicated findings remain unclear; however, the use of venetoclax against cells with ABCG2-mediated resistance may be advantageous. Unexpectedly, the mRNA expression levels of ABCC1 and ABCC2 were significantly lower in HL60/VEN cells compared with HL60 cells (Figure 3). The down-regulation of the ABCC family suggests the potential of venetoclax to reverse resistance mediated by ABCC1 or ABCC2; however, there has been no report on the effects of venetoclax on these substrates as far as we are aware.

In conclusion, the venetoclax-resistant HL60/VEN cells established here displayed up-regulation of the expression and function of ABCB1, in addition to an alteration in the BCL2 family-associated pathway and apoptosis induction. Of note, resistance to other anticancer drug substrates of ABCB1, in addition to venetoclax, was demonstrated in HL60/VEN cells, although the sensitivity to venetoclax was barely affected by ABCB1. These findings highlight the need to monitor cross-resistance and drug–drug interaction caused by the induction of ABCB1 by venetoclax in cancer chemotherapy.

Conflicts of Interest

The Authors declare that no conflicts of interest exist.

Authors' Contributions

Yuko Nakayama: Investigation, data curation, visualization and original draft preparation. Kohji Takara: Data curation, reviewing, editing and supervision. Tetsuya Minegaki: Investigation and data curation. Kazuhiro Yamamoto: Visualization and data curation. Tomohiro Omura: Reviewing and editing. Ikuko Yano: Reviewing, editing and supervision.

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